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PRINCIPAL INVESTIGATOR: John G. Koland, Ph.D.

CONTRACTING ORGANIZATION: University of Iowa
Iowa City, Iowa 52242-1320

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University of Iowa
Iowa City, Iowa 52242-1320

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The role of the ErbB3 protein in breast cancer cell proliferation was examined. In the first year of funding, it was demonstrated that the ErbB3 protein, although possessing a protein tyrosine kinase (PTK) homology domain, is in fact devoid of intrinsic PTK activity. In the second year of funding, we demonstrated the critical dependence of heregulin signaling upon the PTK activity of the ErbB2 protein, and characterized the interaction of ErbB3 with phosphatidylinositol 3-kinase, an enzyme known to be involved in heregulin signaling. In the previous year, we have clarified the role of the Shc protein in the activation of the Ras/mitogen-activated protein kinase (MAPK) pathway. By site-directed mutagenesis, we demonstrated that tyrosine 1325 in the ErbB3 protein serves when phosphorylated as a docking site for Shc, and that this single residue is essential for the activation of the Ras/MAPK pathway. We also began an exhaustive examination of other tyrosine residues in the ErbB3 C-terminus. We generated a series of recombinant ErbB3 proteins in which C-terminal tyrosine residues are substituted with phenylalanine, so that the roles of these sites in the activation of mitogenic signaling via the phosphatidylinositol 3-kinase pathway can be elucidated.

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John T. Kelly 9/29/97

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TABLE OF CONTENTS

INTRODUCTION

Breast cancer cells have been observed to express abnormally high levels of receptor proteins in the ErbB family, which includes the EGF receptor, ErbB2, ErbB3 and ErbB4 (also designated as HER1-HER4, respectively) (1-5). High levels of EGF receptor and ErbB2 expression in tumor cells have been considered indicators of poor prognosis (6). Given that these receptors activate mitogenic signaling pathways, it is possible that they play a role in the abnormal proliferation of breast cancer cells. The polypeptide heregulin (or Neu differentiation factor) (7) is secreted from breast cancer cells (8), and has been shown to activate ErbB2, ErbB3 and ErbB4 receptor proteins (9-12). Indeed, ErbB2 and ErbB3 have been shown to function together as a coreceptor for heregulin (13). Studies completed in the first year of funding had demonstrated that the ErbB3 protein, unlike other ErbB family members, is actually devoid of intrinsic protein tyrosine kinase (PTK) activity. In the second year of funding, these studies were extended to demonstrate that in ErbB2/ErbB3 coreceptors, the ErbB2 protein provides the PTK activity necessary for signal transduction. Also, the yeast two-hybrid system was used to investigate the mechanism of interaction between the ErbB3 protein and the signal-transducing enzyme phosphatidylinositol (PI) 3-kinase.

Efforts in the previous year of funding have focussed on three areas: (i) writing and revising of manuscripts related to the efforts of the second funding period, (ii) examination of the mechanism of activation of the Ras/mitogen-activated protein kinase (MAPK) pathway by ErbB2/ErbB3 heregulin coreceptors, and (iii) further examination of the signaling mechanisms of the ErbB3 protein by the generation of a variety of recombinant ErbB3 proteins in which individual C-terminal Tyr residue phosphorylation sites are substituted with Phe.

BODY

I. Writing and revising of manuscripts related to the efforts of the second funding period

Work in the second funding period yielded experimental results that should be the basis of two significant published manuscripts. The first, describing the role of ErbB2 PTK activity in signal transduction by ErbB2/ErbB3 heregulin coreceptors is in the process of revision. This has required the performance of additional experiments that, as suggested by the reviewers, should ultimately enhance the rigor of our findings. One series of experiments has addressed the issue of whether in the immunoprecipitation of one ErbB heregulin coreceptor constituent (e.g. ErbB3), the other coreceptor constituent (e.g. ErbB2) was co-precipitated. If coreceptor constituents did co-precipitate under the conditions of our study, our investigation of the phosphorylation of the individual ErbB proteins would be subject to dispute. Fortunately, through a series of control experiments, we were able to demonstrate that co-precipitation of the involved ErbB proteins was negligible. Similar experiments were necessary to strengthen our argument that certain phosphoproteins precipitated by Shc-specific antiserum were indeed the Shc proteins and not Shc-associated proteins. The preparation of a second manuscript describing the mechanism of interaction between the ErbB3 protein and the p85 subunit of phosphatidylinositol (PI) 3-kinase has also required the performance of additional experiments. Significantly, we have provided mammalian cell culture experiments to bolster the results of our yeast two-hybrid system analysis. Here, we have shown conclusively that phosphorylation of C-terminal Tyr residues within Tyr-Xaa-Xaa-Met (YXXM) sequence motifs is essential for the interaction of the p85 protein with the ErbB3 protein. This was accomplished by substituting by site-directed mutagenesis each of the six Tyr residues within YXXM motifs in the ErbB3 C-terminus with Phe (see **III**). When expressed in cultured fibroblasts expressing endogenous ErbB2 protein, this recombinant ErbB3 protein with six Tyr→Phe amino acid substitutions failed to associate with the p85 protein upon stimulation of the cultured cells with heregulin. The performance of these additional experiments in the previous funding period should represent the successful completion of our two studies of ErbB3 signal transduction.

II. Examination of the mechanism of activation of the Ras/mitogen-activated protein kinase (MAPK) pathway by ErbB2/ErbB3 heregulin coreceptors

In the critique of the previous progress report, it was suggested that in the subsequent funding periods it would be prudent for us to pursue the investigation of the signal transduction mechanisms of ErbB3. In line with this suggestion, we have in the previous funding period examined two aspects of ErbB3 signaling: (i) the role of the Ras/MAPK pathway as described here, and (ii) the role of the PI 3-kinase pathway as described below (see **III**). In order to investigate the role of the Ras/MAPK pathway in ErbB3 signaling, which is frequently mediated via Shc adapter proteins, we first generated by site-directed mutagenesis a recombinant ErbB3 protein in which the single C-terminal Tyr residue (Tyr-1325) found within a consensus Shc-binding site motif, Asn-Pro-Xaa-Tyr (NPXY), was substituted with Phe. This mutant protein (ErbB3-Y/F) and the parental wild-type ErbB3 protein (ErbB-WT) were individually expressed in cultured NIH-3T3 fibroblasts via the transfection of cDNAs in the mammalian expression vector pcDNA3 (see Fig. 1A). These NIH-3T3 fibroblasts expressed endogenous ErbB2 protein (see Fig. 1B), such that upon introduction of ectopic ErbB3, heregulin coreceptors were formed. Upon stimulation of these cells with heregulin, both ErbB3-WT and ErbB3-Y/F were phosphorylated in addition to the endogenous ErbB2 protein (see Fig. 1). Experiments characterizing the potential interactions of Shc proteins with the wild-type and mutant ErbB3 proteins were next performed. Figure 2A shows that roughly equivalent levels of each isoform of the Shc protein were present across the cell lines. In subsequent immunoprecipitation experiments, only the ErbB3-WT protein was seen to associate with Shc, and this association was enhanced by prior stimulation of the cells with heregulin (see Fig. 2B). Hence, the heregulin-stimulated and phosphorylated ErbB3 protein interacted strongly with the Shc protein(s), and this interaction appeared to be mediated by Tyr-1325 in the ErbB3 C-terminus. Further experiments showed that in cells expressing ErbB3-WT each of the three Shc isoforms was phosphorylated in response to heregulin, and that heregulin induced a strong Shc/Grb2 association (see Fig. 2C). The Tyr→Phe substitution in ErbB3-Y/F markedly reduced the levels of Shc phosphorylation and Shc/Grb2 association seen in response to heregulin.

Because Shc phosphorylation and Shc/Grb2 association are believed to result in the activation of the MAPK isoforms p42 and p44, we next examined the potential activation of MAPK activity by the two ErbB3 receptor proteins. As

demonstrated by either by gel-shift assays (see Fig. 3A) or immune-complex kinase assays (see Fig. 3B), cells expressing ErbB3-WT showed robust MAPK activation in response to heregulin. However, in cells expressing the ErbB3-Y/F protein, negligible MAPK activation was evident. Together these results showed that the substitution of Tyr-1325 in the ErbB3 C-terminus with Phe rendered ErbB2/ErbB3-Y/F coreceptors incapable of activating the Ras/MAPK pathway, presumably due to their failure to recruit and phosphorylate the Shc protein(s). Additional experiments confirmed that this amino acid substitution did not affect the ability of the ErbB3 protein to interact with the p85 subunit of PI 3-kinase in response to heregulin (see Fig. 4), an interaction that would likely be mediated by the phosphorylation of distinct ErbB3 Tyr residues (see **III**).

Given the failure of ErbB2/ErbB3 coreceptors incorporating the ErbB3-Y/F protein to activate the MAPK pathway, it was possible to examine the role of this pathway in mitogenic signaling. Experiments were conducted in which the rate of DNA synthesis in the fibroblasts was assayed after challenge with varying concentrations of heregulin (see Fig. 5). Cells expressing ErbB3-WT showed a high basal mitogenesis relative to mock-transfected cells and cells expressing ErbB3-Y/F. Whereas DNA synthesis was significantly stimulated by increasing heregulin concentrations in cells expressing ErbB3-WT, this stimulation was reduced in cells expressing ErbB3-Y/F and absent in mock-transfected cells. Together, these results indicated that activation of the Ras/MAPK pathway via ErbB2/ErbB3 heregulin coreceptors contributes to a high basal rate of mitogenesis in fibroblasts expressing these coreceptors, and is in part responsible for heregulin-stimulated mitogenesis in these cells. It was considered that other signaling pathway(s) activated by the ErbB2/ErbB3 coreceptor, for example the PI 3-kinase pathway, might have been responsible for the residual heregulin-stimulated DNA synthesis seen in cells expressing the ErbB3-Y/F protein.

III. Further examination of the signaling mechanisms of the ErbB3 protein by the generation of recombinant ErbB3 proteins in which individual C-terminal Tyr residue phosphorylation sites are substituted with Phe

The results described above indicated the utility of the site-directed mutagenesis approach in the investigation of the signaling mechanisms of ErbB receptor proteins. Because the ErbB3 protein is unique among EGF receptor/ErbB family members in that its C-terminal phosphorylation domain contains a large number (12) of candidate Tyr residue phosphorylation sites, we have endeavored

to explore the roles of these various Tyr residues by Tyr→Phe substitutions as described above. Efforts of the previous funding period have been directed towards the six C-terminal Tyr residues within YXXM motifs that are candidate PI 3-kinase binding sites. Because the p85 regulatory subunit of PI 3-kinase contains two SH2 (Src homology 2) domains, each independently capable of binding to phosphorylated YXXM motifs, a variety of scenarios for the interaction between ErbB3 and p85 might be envisioned, some involving multiple ErbB3 YXXM motifs. Hence, we considered it would be necessary to generate a large number of Tyr→Phe mutants in which various combinations of the six YXXM motifs were altered (see Fig. 6). First, a cDNA encoding a mutant ErbB3 protein (B3-6FXXM) in which all of the six Tyr residues in YXXM motifs were substituted with Phe was constructed. This cDNA served as the template for the construction of cDNAs encoding a series of add-back mutants in which individual Phe residues were restored to Tyr. Finally, recognizing that interactions between p85 and ErbB3 could be potentiated by tandem YXXM motifs, we created cDNAs encoding double add-back mutants, in which pairs of neighboring YXXM motifs were restored by Phe→Tyr substitutions. While it is recognized that a variety of other YXXM motif mutants could be envisioned, the set of 11 mutant proteins for which cDNA expression vectors have now been constructed (see Fig. 6) should allow us to begin our investigation of the mechanism of the ErbB3/PI 3-kinase interaction. We have preliminary evidence (data not shown) that the B3-6FXXM mutant ErbB3 protein, when co-expressed in COS7 cells with the ErbB2 protein, is phosphorylated on Tyr residues in response to heregulin, but fails to interact with the p85 protein. Hence, we believe that these mutant cDNAs can be successfully exploited in the examination of the mechanism of interaction of PI 3-kinase with ErbB2/ErbB3 heregulin coreceptors. Questions of particular interest are: (i) are specific YXXM motifs critical for the interaction of p85 with ErbB3; (ii) do tandem YXXM motifs enhance this interaction; (iii) does the presence of six YXXM motifs further enhance the interaction; and (iv) what are the consequences for the activation of downstream PI 3-kinase effectors such as protein kinase B (PKB/Akt). Just as we have used the ErbB3-Y/F mutant to investigate the role of the Ras/MAPK pathway (see **II**), we also hope to use the B3-6FXXM mutant to investigate the role of PI 3-kinase in mitogenic signaling by ErbB2/ErbB3 coreceptors. In this context, it would be useful to create an additional mutant receptor protein in which the identified Shc binding site Tyr residue and the six Tyr residues in YXXM motifs are substituted with Phe.

CONCLUSIONS

Knowledge of the mechanism of ErbB2/ErbB3 coreceptor signaling is crucial to our understanding of the control of breast cancer cell proliferation, as many of the cultured breast cancer cells examined to date express abnormally high levels of both of these receptor proteins. The heregulin polypeptide, which is an activating ligand for the ErbB2/ErbB3 coreceptor, is known to be expressed and secreted by breast cancer cells, and could therefore be critical to the proliferation of those breast cancer cells expressing the coreceptor. However, because of the complexity of heregulin/heregulin coreceptor interactions, our understanding of the signaling mechanisms of heregulin coreceptors is yet limited.

Studies completed in the third year of research have begun to elucidate the role of the Ras/MAPK and Shc proteins in signaling by the ErbB3 protein. It appears that under some conditions the ErbB3 protein can play a dominant role in the recruitment of signaling molecules to ErbB2/ErbB3 coreceptors. This contrasts the dominant role played by the ErbB2 PTK in the phosphorylation of coreceptor constituents and associated signaling molecules. The ErbB3-Y/F mutant has been shown to be useful for investigations of the contribution of the Ras/MAPK pathway to mitogenic signaling, and could be exploited in future studies of the mechanisms of cellular transformation. Additionally, we have generated a series of cDNAs encoding mutant ErbB3 receptor proteins that will be applied in future investigations of the mechanism of activation of PI 3-kinase and the contribution of this pathway to mitogenic signaling by ErbB2/ErbB3 heregulin coreceptors and their transforming potentials.

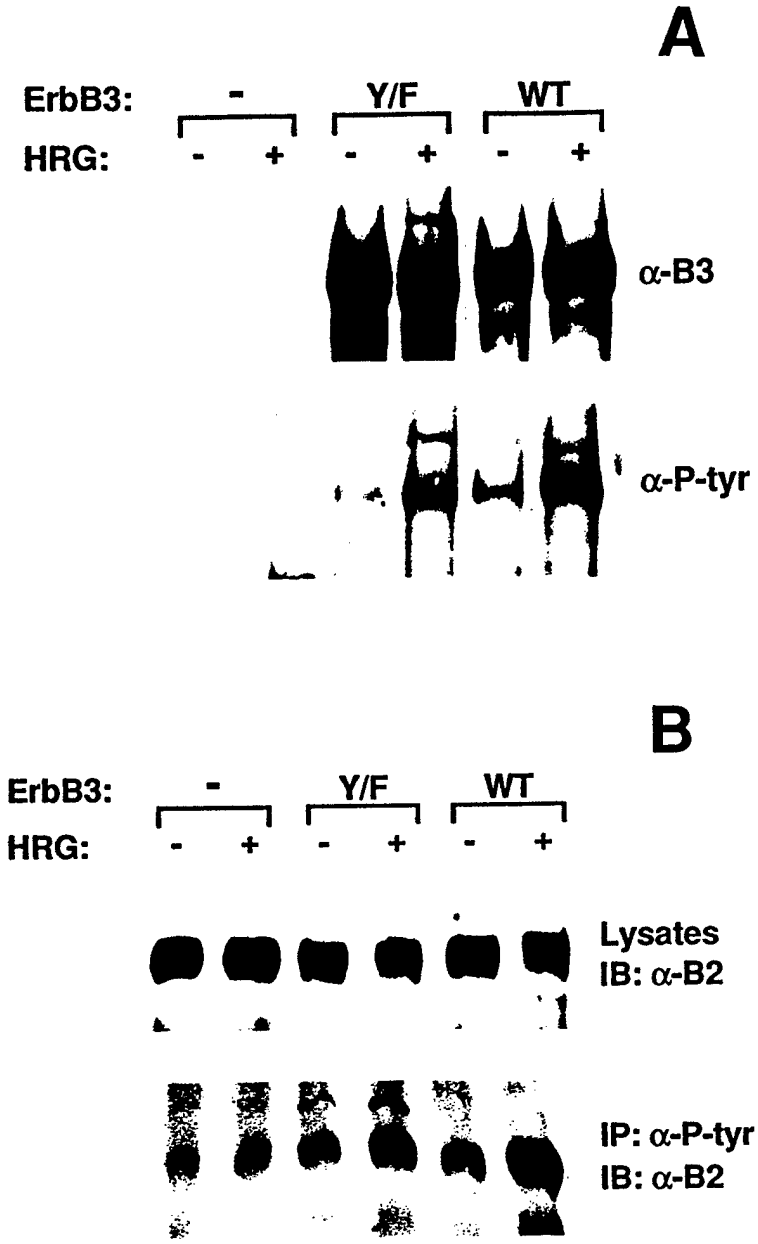

FIG. 1. **Expression and heregulin-mediated phosphorylation of ErbB2 and ErbB3 in stably transfected NIH-3T3 cell lines.** NIH-3T3 cells were transfected with either the parent pcDNA3 expression vector (—), or pcDNA3 incorporating wild-type (WT) and Tyr-1325→Phe mutant (Y/F) ErbB3 cDNAs. Mock-transfected cells and cells expressing ErbB3-Y/F and ErbB3-WT were serum-starved and stimulated with vehicle or 1 nM heregulin-β1. *A*, cell lysates containing 1 mg of protein were immunoprecipitated with an ErbB3-specific antibody, and the immunoprecipitates subjected to SDS-PAGE and immunoblotting with either ErbB3-specific antibody (α-B3) or antiphosphotyrosine antibody (α-P-tyr). *B*, alternatively, cell lysates were blotted with ErbB2-specific antibody (α-B2) or immunoprecipitated with a phosphotyrosine-specific antibody (α-P-Tyr) and then immunoblotted with ErbB2-specific antibody.

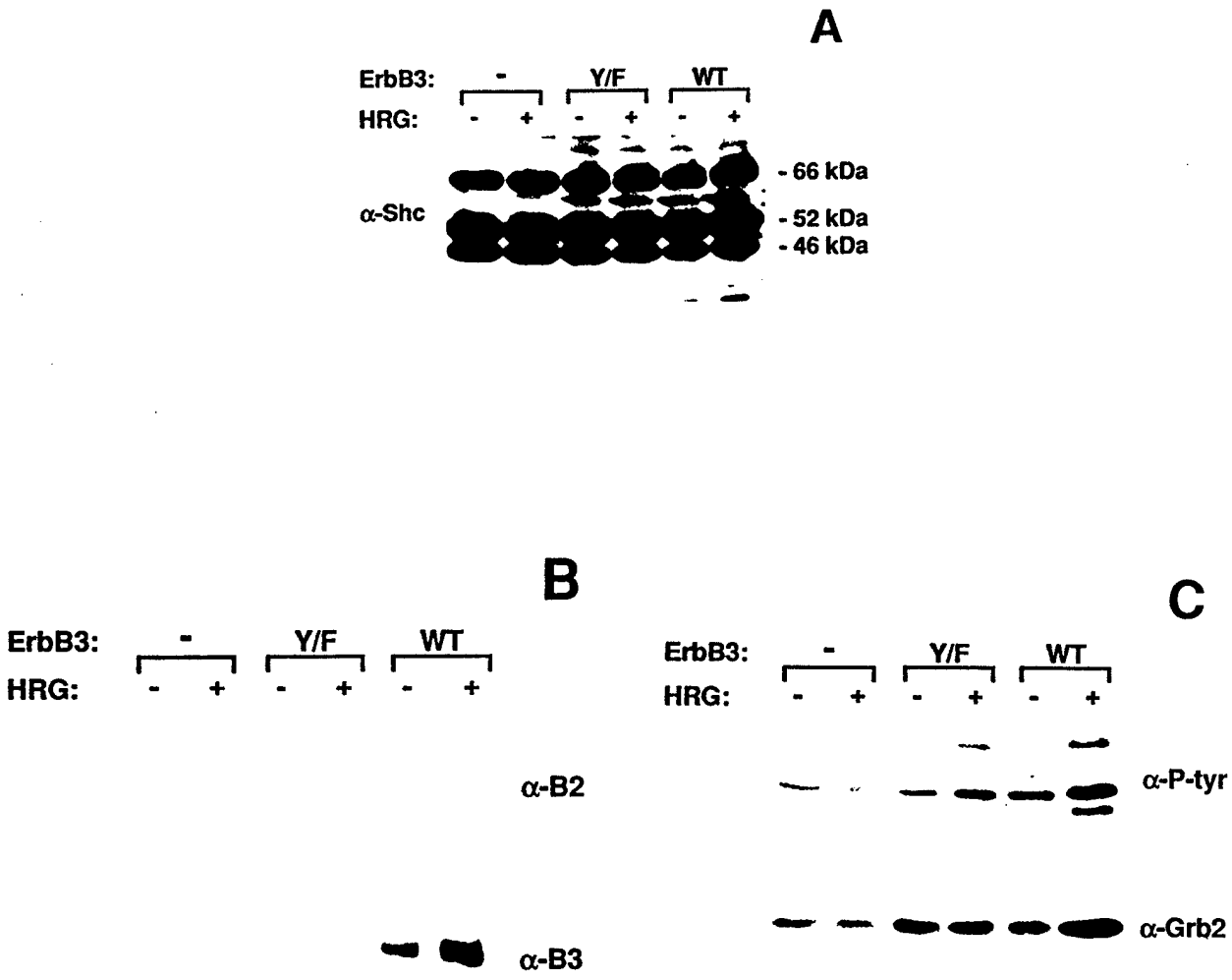

FIG. 2. **Heregulin-stimulated ErbB3/Shc association, Shc phosphorylation, and Shc/Grb2 association.** NIH-3T3 cells transfected with vector, ErbB3-WT or ErbB3-Y/F cDNAs were treated as described in Fig. 1. *A*, lysates from cells stimulated with heregulin or control vehicle were probed with a Shc-specific antibody (α-Shc). All three isoforms of Shc (p46, p52, p66) were evident. *B*, Shc was immunoprecipitated from the lysates with a Shc-specific antibody, and the immunoprecipitates were immunoblotted with either ErbB2-specific (α-B2) or ErbB3-specific (α-B3) antibody. *C*, Shc immunoprecipitates were also immunoblotted with the antiphosphotyrosine-horse radish peroxidase conjugate (α-P-tyr) or a Grb2-specific antibody (α-Grb2).

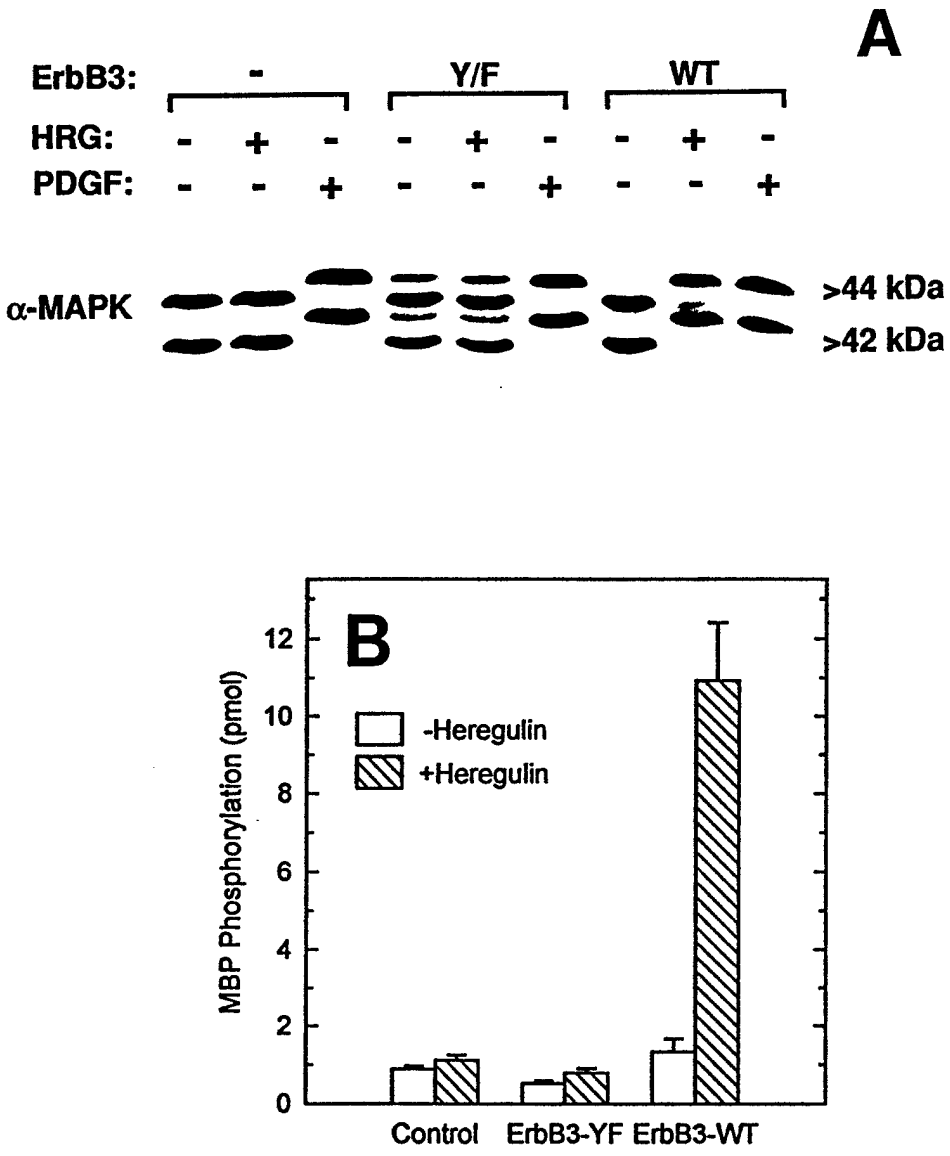

FIG. 3. **Heregulin-stimulated activation of mitogen-activated protein kinase.** *A*, cells stimulated with heregulin (1 nM), platelet-derived growth factor (50 ng/ml) or vehicle were lysed as described in Fig. 1. Cell lysates containing 70 μg of total protein were subjected to a gel mobility shift assay of MAPK activation. Both the p42 and p44 isoforms of MAPK are indicated. The appearance of more slowly migrating bands in cells transfected with wild-type ErbB3 cDNA in response to heregulin and in all three cell lines in response to platelet-derived growth factor indicated the activation of the MAPK isoforms. *B*, alternatively, MAPK was immunoprecipitated from heregulin-stimulated and control cells, and the immunoprecipitates were subjected to *in vitro* MAPK assays with MBP and [γ^{32}P]ATP as substrates. Error bars represent the standard error of three independent experiments.

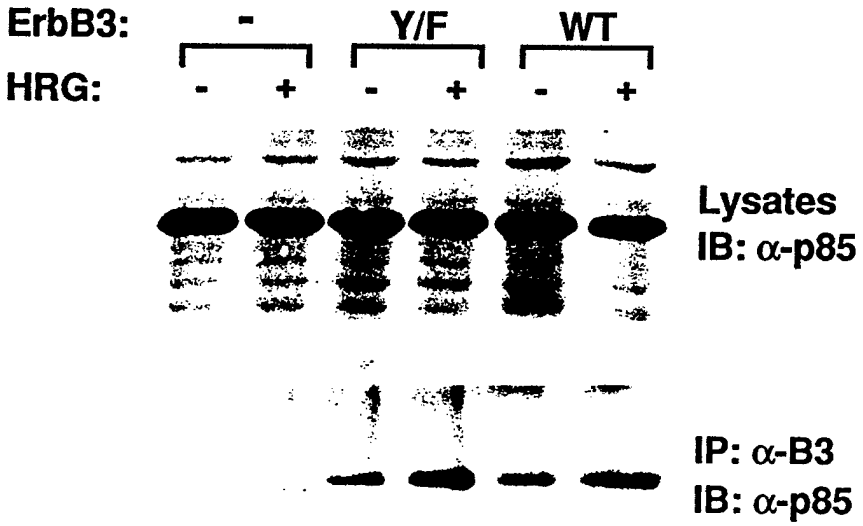

FIG. 4. **Association of PI 3-kinase with ErbB3-WT and ErbB3-Y/F in stably transfected NIH-3T3 fibroblasts.** Transfected cells were treated as described in Fig. 1. Lysates from vehicle- or heregulin-stimulated cells were probed with an antibody recognizing the p85 regulatory subunit of PI 3-kinase (α-p85). Cell lysates containing 1 mg of protein were also immunoprecipitated with an ErbB3-specific antibody (α-B3), and the immunoprecipitates subjected to SDS-PAGE and immunoblotting with p85-specific antibody.

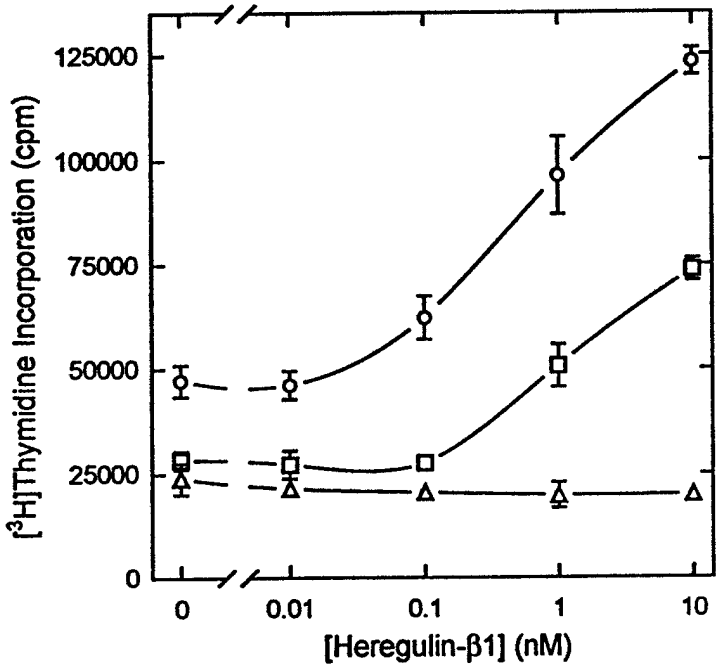

FIG. 5. **Heregulin-stimulated [^{3}H]thymidine uptake by NIH-3T3 cells expressing ErbB3-WT and ErbB3-Y/F.** Transfected cells were serum-starved overnight followed by treatment with varying concentrations of heregulin for 18 h. [Methyl-^{3}H]thymidine was then added to the stimulation medium, and its incorporation into DNA determined after 4 h. NIH-3T3 cells were transfected with pcDNA3 (Δ), pcDNA3-B3-Y/F (❑), pcDNA3-B3-WT (O). Error bars represent the standard deviation of triplicate assays.

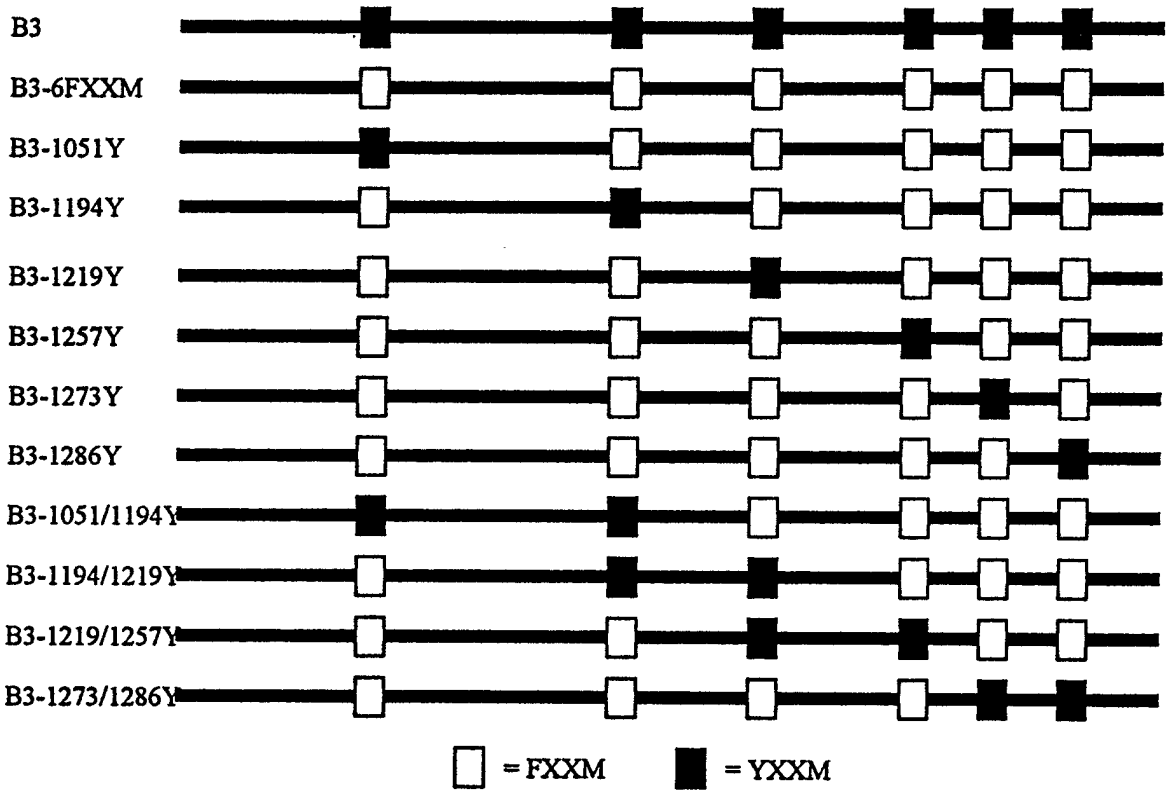

FIG. 6. **Schematic structures of ErbB3 proteins incorporating Tyr→Phe amino acid substitutions in YXXM motifs.** The structure of the C-terminal phosphorylation domain of the ErbB3 protein is shown with Tyr residues within YXXM motifs indicated as closed boxes and substituted Phe residues indicated as open boxes. B3 designates the wild-type protein, and the amino acid numbering of the afffected residues is also indicated.

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